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14. ABSTRACT

The goal of the project is to determine the effects on metastasis of deleting or inhibiting the receptor for interleulin-4 from breast tumor cells. We proposed three aims: [1] Establish and characterize mammary tumor lines with the receptor for IL4 knocked down. [2] Determine the *in vivo* growth characteristics of IL4R-knockdown cells using spontaneous and experimental metastasis models; [3] Determine the therapeutic efficacy of systemic IL4 neutralizing antibody in combination with chemotherapy in a spontaneously-metastasizing breast cancer model. We have successfully achieved aim 1 for 1 of the 2 cell lines originally proposed and are continuing our efforts with the second cell line. In vitro assays have indicated that IL4 receptor indeed functions to promote proliferation and survival of the mouse mammary tumor cell line, 4T1. Further, signaling through IL4R appears to promote survival after exposure to paclitaxel. We anticipate that the in vivo assays, which are just beginning, will confirm this role of IL4R.If confirmed, then we would propose that IL4R blockade be used clinically in combination with chemotherapy to reduce the occurrence of relapse.

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INTRODUCTION

Cancer cells that persist after chemotherapy are especially dangerous to patients as they can seed new tumor outgrowths. Interleukin-4 (IL4), a cytokine known primarily for its immune system functions, has been proposed as a survival factor for breast cancer cells, and thus disabling its function provides a possible method to ablate these insidious tumor cells. The hypothesis we wished to test therefore was :**Blocking IL4 signaling will attenuate breast tumor cell survival and, in combination with chemotherapy, will promote disease eradication.** We proposed three aims to test this hypothesis: [1] Establish and characterize mammary tumor lines with the receptor for IL4 knocked down. [2] Determine the *in vivo* growth characteristics of IL4R-knockdown cells using spontaneous and experimental metastasis models; [3] Determine the therapeutic efficacy of systemic IL4 neutralizing antibody in combination with chemotherapy in a spontaneously-metastasizing breast cancer model.

BODY

The goal of the project is to determine the effects on metastasis of deleting or inhibiting the receptor for interleulin-4 from breast tumor cells. The primary method chosen for deleting expression of the gene was shRNA-mediated knockdown. We proposed to use 2 different murine mammary tumor cell lines for this work, as it was considered very important that studies not be limited to a single cell line. Furthermore, within each cell line, we proposed using several clones with different shRNA targeting sequences to minimize the possibility of artifact or off-target effects. In the 4T1 cell line, we have successfully generated 2 knockdown clones for each of 2 different targeting sequences, and one clone with a third targeting sequence. However, the PyVT-R221A cell line has proven resistant to our manipulation with only minimal (less than 30%) knockdown in one clone. This is despite screening a large number of clones (~25). The problems with obtaining efficient knockdown have compromised our ability to do the *in vitro* and *in vivo* assays described in tasks 2 and 3. On the advice of a colleague who had similar difficulties with achieving efficient target knockdown, we have now obtained a new shRNA system (shRNAmir), which based on experience with other recalcitrant targets, greatly improves knockdown efficiency. We therefore requested an extension for this project of 1 year to ensure that all experiments can be appropriately completed.

Following, we detail the progress for each task originally defined in our statement of work.

Task 1: Obtain regulatory approval for animal experiments

All regulatory approvals were received before the commencement of the project.

Task 2: Generate and characterize mammary tumor cell lines without IL4R

2a: Use commercially available lentiviral shRNA particles to infect cultures of 4T1 and PyVT-R221A cells and select clones with puromycin.

2b: Analyze levels of knockdown in various clones by RT-PCR and western blotting

This task, which was scheduled to take 2 months, actually took 4 months to complete for the 4T1 cell line; and has yet to be completed for the PyvT-R221A cells.

Lentiviral shRNA particles generated against 4 different sequences of murine IL4Ra (Sigma, St Louis, Mo) were used to infect cultures of both 4T1 and PyVT-R221A cells. After selection with puromycin, multiple colonies were obtained for both cell lines and with 3 of the 4 shRNA sequences. One of the sequences never resulted in puromycin-resistant colonies. Colonies were picked and expanded in puromycin-containing medium and then stocks were frozen. Lysates were prepared from individual colonies and used for western blotting of IL4Ra. RNA was also prepared and analyzed by RT-PCR using IL4Ra-specific primers. Of 30 4T1 clones examined (10 for each of the three successful targeting sequences), 5 showed a protein knockdown of greater than 50%. The top three, each representing a different targeting sequence were selected for further use. These clones: 188.2; 189.8 and 190.4 showed knockdown of 75-90% at the protein level, which was confirmed at the transcript level. Two control clones which had been infected with a scrambled non-targeting shRNA were also selected for further use. As expected, these control clones (C3 and C5) showed no change in IL4Ra level. Figure 1 shows both the control and knockdown clones as analyzed at the protein level using a specific antibody for IL4Rα.

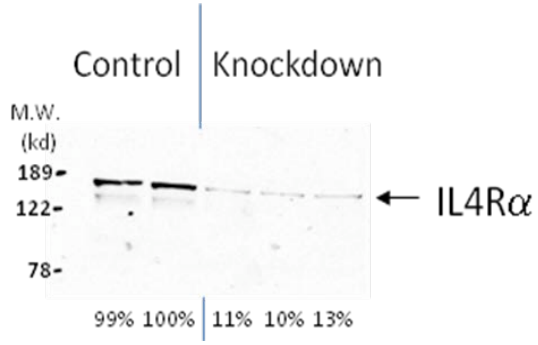

Fig 1: Successful knockdown of IL4Ra expression in 3 different 4T1 clones compared to two control clones. Numbers along the bottom indicate percentage of protein expression relative to one of the controls, as calculated using ImageJ software.

Multiple colonies(>25) of PyVT-R221A cells were expanded and similarly tested by both western blotting and RT-PCR. Despite showing puromycin resistance, none of the clones showed more than 30% knockdown when analyzed. This level is not regarded as useful. Since we believe it important to have more than one cell line used for the studies described in this project, we have decided to obtain a new type of lentiviral shRNA (shRNA-mir), which our colleagues have used for other targets and find to be vastly superior to what we have used previously. We are currently working on generating PyVT-R221A knockdown clones using this technology.

2c: Analyze proliferation and apoptosis rates in vector control vs IL4Ra knockdown (KD) clones, both basal and post-paclitaxel exposure.

Baseline proliferation was not different amongst the various 4T1 control and IL4Ra-KD clones, as assessed by MTT assay. However, control clones responded to increasing concentrations of exogenous recombinant IL4 with increased proliferation, but this response was not observed in the 4T1 IL4R-KD clones (Fig 2). This result was expected since the proliferative activity of IL4 requires signaling through IL4Ra. Interestingly, the IL4Ra-KD clones retained sensitivity to increasing concentrations of IL13 (Fig 2). IL13 is a related cytokine to IL4 and most of its signaling is also thought to require IL4Ra. There is a second version of an IL13 receptor that does not contain IL4Ra, however whether is a functional receptor or merely acts as a decoy is not clear from the literature. Our results suggest the second IL13 receptor may be functional, at least in 4T1 cells.

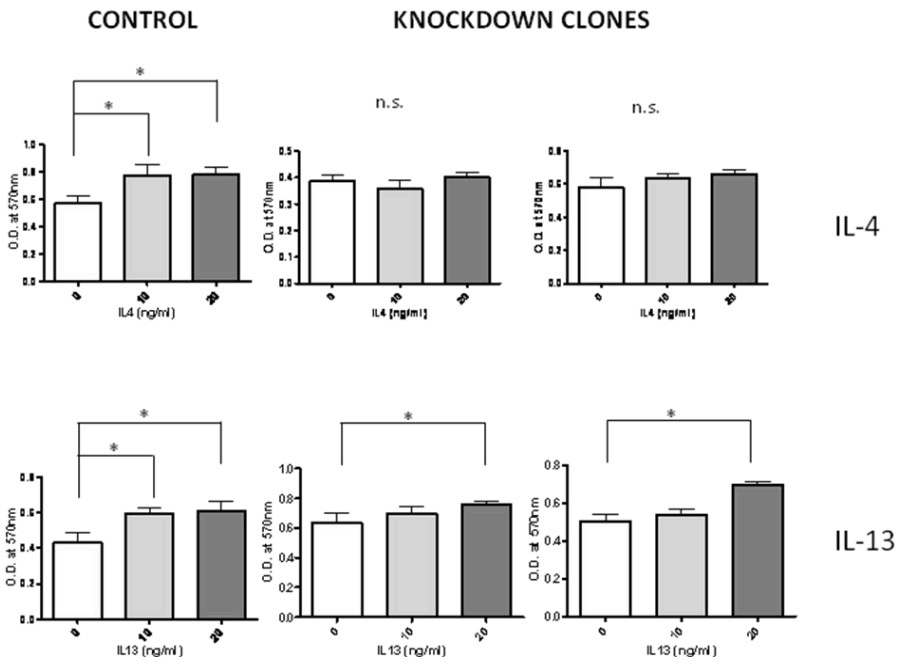

Fig 2: MTT assays showing response of control, but not knockdown 4T1 clones to increasing concentrations of recombinant murine IL4. The knockdown clones retain sensitivity to the higher concentration of recombinant IL13.* = $p<0.05$, using ANOVA with Dunnett's post-hoc test. n.s. = not significant.

To assess apoptosis and proliferation post-paclitaxel, we initially used MTT assays and varying doses of paclitaxel. 4T1 cells are sensitive, but not at low doses. Using MTT assays, a dose of 5 μM paclitaxel was identified as the IC50 for the control clones, however 2 of 3 of the IL4Ra-KD clones required 1-2 μM to kill 50% of the cells. This is in the absence of exogenous IL4 or IL13.

2d: Analyze clonogenicity using soft agar assays of vector control versus KD clones, both basal and post-paclitaxel exposure.

Under basal conditions, clonogenic survival was similar amongst control and IL4Ra-KD clones. When recombinant IL4 was added, there was an increase in colony size (indicating a proliferative effect) and colony number (indicating a survival effect) in the control clones (Fig 3). IL4Ra-KD clones showed no change in size, although there was a small increase in colony number in some wells leading to high variability (Fig 3). This may suggest that survival is very sensitive to signaling through IL4Ra, and even with only 10-25% IL4Ra protein remaining, this is sufficient to confer increased survival ability in response to IL4.

The addition of paclitaxel to the clonogenic survival assays was not as straightforward as anticipated, as the doses identified by MTT assays as IC50s allowed for no surviving

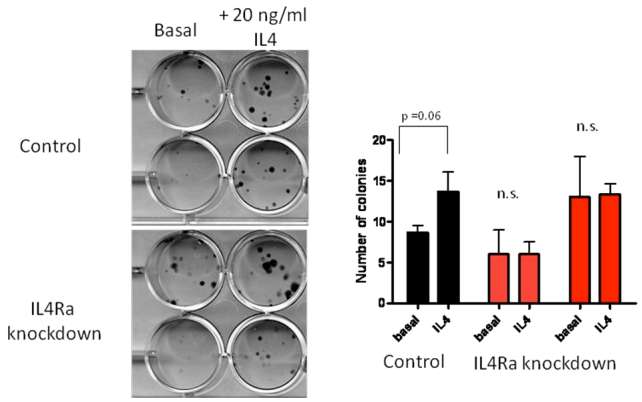

Fig 3: Exogenous IL4 increases size and number of colonies of control 4T1 cells in a clonogenic assay. On the left are photographs of sample wells, while the results for colony number are graphed on the right. The statistical significance was determined using student's t-test for each control and knockdown clone comparing basal versus IL4. Control clones approached significance (p=0.06), whereas knockdown clones were not different. n.s. = not significant.

colonies. We thus re-did dose-response curves to paclitaxel using clonogenic survival as the readout. In the absence of IL4, 0.5 μM was identified as the dose irrespective of IL4Ra-KD status. In the presence of IL4, control clones exposed to paclitaxel demonstrated more colonies than did IL4Ra-KD clones under the same conditions. However, after statistical analysis the differences were not found to be significant for any clone due to a high degree of variability. Our data suggests that signaling through IL4Ra does appear to confer a survival advantage, both at baseline and after exposure to paclitaxel, but repetition will be required to fully demonstrate this effect. If our hypothesis holds true, we believe that inhibitors of the IL4Ra pathway may be clinically useful to prevent survival of tumor cells during chemotherapy.

Task 3: Determine in vivo growth capability of control vs KD clones

3a: Perform orthotopic implantation of 4T1 control and KD cells into 6-week old female BALB/c mice (8 per line). Sacrifice after 5 weeks and harvest tissues.
We have decided to mix the control clones together to make a polyclonal control, and similarly mix the KD clones together to make a polyclonal KD cell inoculum. The polyclonal cell populations will be used for all in vivo experiments.
The mice have been purchased and are currently acclimatizing in our animal house. These injections are scheduled to occur on Sept 1.
3b: Perform tail vein injection of 4T1 control and KD cells into 6-week female BALB/c mice (8 per line).
The mice have been purchased and are currently acclimatizing in our animal house. These injections are scheduled to occur on Sept 2.
3c: Perform intrasplenic injection of 4T1 control and KD cells into 6-week female BALB/c mice (8 per line).
The mice have been purchased and are currently acclimatizing in our animal house. These injections are scheduled to occur on Sept 2.
3d: Perform intra-tibial injections of 4T1 control and KD cells into 6-week old female BALB/c mice.
These injections are scheduled for the beginning of October.
3e-g : Injections of PyVT-R221A control and KD clones.
These injections must be postponed until we obtain clones with satisfactory levels of IL4Ra knockdown.
3h-j: Analysis of tissue
These tasks will begin once the injected mice reach the timepoint for euthanasia i.e. will begin mid-September when the tail vein and splenic injection models have been completed.

Task 4: Determine efficacy of systemic IL4 neutralization +/- chemotherapy in 4T1 model.
This task has been postponed until Jan 2011 to give time to complete other mouse studies.

Task 5: Write and submit manuscript describing these studies.
This task cannot be completed until all the studies described in previous tasks have ended. We have however begun to collate the *in vitro* data with the 4T1 clones as one section of the manuscript.

KEY RESEARCH ACCOMPLISHMENTS

[1] Have successfully obtained multiple IL4Rα-knockdown clones of the murine mammary tumor cell line, 4T1.

[2] *In vitro* assays indicate that IL4Rα is required for the proliferative effect of IL4, but not of a closely related cytokine IL13.
[3] Knockdown of IL4Rα appeared to inhibit the survival of 4T1 cells following paclitaxel treatment indicating that IL4R blockade may be a clinically useful approach to reduce the occurrence of post-treatment relapse.

REPORTABLE OUTCOMES

Generation of stable clones of IL4Ra-knockdown 4T1 cells

CONCLUSION

Although we are as yet limited to *in vitro* data with a single cell line, our results indicate that IL4 is a survival factor for breast cancer cells. This is especially relevant in the setting of chemotherapy, where cells that survive may be the seeds for a recurrence of the cancer. If the data from our *in vivo* studies look similar to the *in vitro* data, then we will strongly advocate for the clinical testing of IL4 blockade in combination with chemotherapy as a reasonable approach to prevent disease recurrence.